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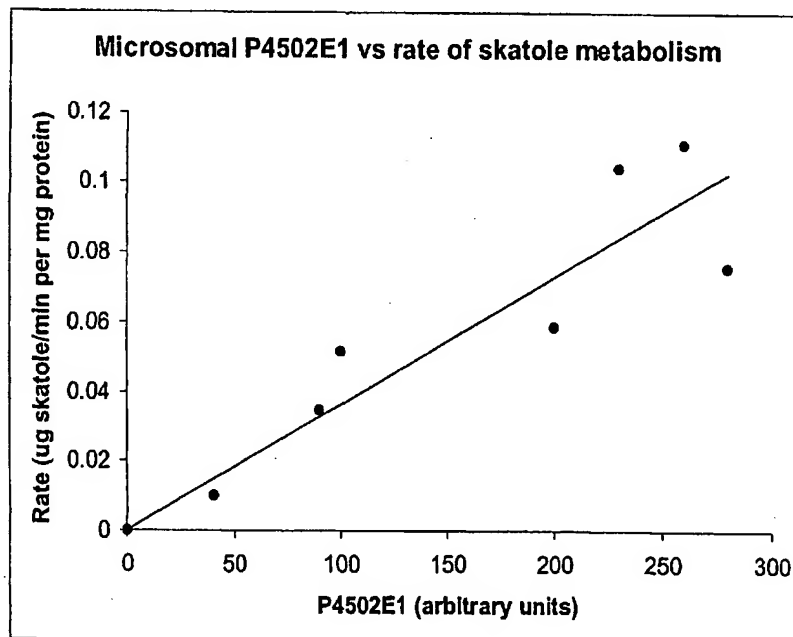
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(54) Title: DIAGNOSTIC ASSAY FOR BOAR TAINT



(57) Abstract: The present invention discloses a link between the level of cytochrome P450 isoform P4502E1 and the level of skatole. A polynucleotide encoding the sequence for P4502E1 and a polynucleotide encoding the sequence for P4502E1 and a polynucleotide encoding the sequence for P4502E1 for one pig with high skatole is provided. An assay to identify pigs with a genetic predisposition is also provided.

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1 DIAGNOSTIC ASSAY FOR BOAR TAIN

2

3 The present invention relates to genetic markers,
4 which may include a functional mutation for pigs
5 exhibiting desirable flavour properties. In
6 particular, the present invention provides an assay
7 to screen pigs for boar taint and its associated
8 flavours. Generally pigs having low boar taint
9 levels will be positively selected, but it is also
10 possible to identify animals having unacceptably high
11 boar taint levels.

12

13 Background

14

15 Boar taint - economic impacts

16

17 "Boar taint" is a strong perspiration-like, urine-
18 like unpleasant odour given off upon heating or
19 cooking of meat from some entire (uncastrated) male
20 pigs. The off-odours and off-tastes, commonly known
21 as "boar taint", are objectionable to consumers. In

1 the United States carcasses tainted by boar odour are
2 either condemned or subject to restricted use by
3 United States Department of Agriculture meat
4 inspectors. EU law (Council Directive 91/497/EEC,
5 which has been implemented in Britain through the
6 Fresh Meat (Hygiene and Inspection) Regulations
7 1992)) states that animals over 80 kg carcass weight,
8 excluding the head, should be screened for boar
9 taint, but no method is specified.

10

11 The most effective method, to date, for preventing
12 "boar taint" is to castrate (i.e., remove the testes
13 of) young male pigs. Castration of young male pigs
14 is widely practised in pig production systems in
15 North America and Europe. However, as outlined
16 below, there are production advantages of using
17 entire male pigs. Entire male pigs are used
18 extensively in pig production in the United Kingdom
19 and also in Denmark, Australia and parts of Spain.
20 Other measures taken to reduce the risk of boar taint
21 include slaughtering entire male pigs at an earlier
22 age than castrated males.

23

24 Pig production systems that involve castration of
25 young male pigs suffer economic losses and other
26 disadvantages. These economic losses are
27 attributable to lost opportunities to access the
28 superior performance, especially feed conversion, of
29 intact males and the inferior nature of carcasses
30 from castrates (barrows) (see for example: Allen, P.,
31 Riordan, P.B., Hanrahan, T.J. and Joseph, R.L. 1981.

1 Production and quality of boar and castrate bacon.
2 *Irish J. Sci. Technol.* 5, 93-104; Wood, J.D. and
3 Riley, J.E. 1982. Comparison of boars and castrates
4 for bacon production. 1. Growth data, and carcass and
5 joint composition. *Animal Production* 35, 55-63;
6 Ellis, M., Smith, W.C., Clark, J.B.K. and Innes, N.
7 1983. A comparison of boars, gilts and castrates for
8 bacon manufacture. 1. on farm performance, carcass
9 and meat quality characteristics and weight loss in
10 the preparation of sides for curing. *Animal*
11 *Production* 37, 1-9). If the problem of boar taint
12 were overcome, raising boars rather than castrates
13 would have considerable economic advantages.
14 Although boars and castrates gain weight at
15 equivalent rates, boars produce carcasses containing
16 20-30% less fat. Boars also utilise feed more
17 efficiently than barrows (10% less feed consumed per
18 unit of body weight). Since feed represents the
19 major cost in pig production, raising boars for pork
20 would have significant economic advantages.
21
22 Castration not only produces animals with inferior
23 carcass characteristics and a less efficient feed
24 conversion, but is also bad for the pig's welfare.
25 Adverse animal welfare considerations include the
26 pain associated with castration, the loss of 'normal'
27 behaviour and the risk of infection.
28
29 In conclusion, there is a need for methods to prevent
30 or determine predisposition to boar taint, that do
31 not require castration of young pigs.

1 Boar taint

2
3 Boar taint is associated with elevated levels of
4 androstenone (5 α -androst-16-en-3-one), indole and
5 skatole (3-methyl-indole) See Patterson, R.L.S.
6 (1968) 5 α -androst-16-ene-3-one:-compound responsible
7 for taint in boar fat. *J. Sci. Food Agric.* 19: 31;
8 Bonneau, M., Le Denmat, M., Vaudelet, J.C., Veloso
9 Nunes, I.R. Mortensen, A.B. and Mortensen, H.P (1992)
10 Contribution of fat androstenone and skatole to boar
11 taint: II Eating quality of cooked ham. *Livest. Prod.*
12 *Sci.* 32, 81-88; see also Claus et al. 1994.
13 Physiological aspects of androstenone and skatole
14 formation in the boar - a review with experimental
15 data. *Meat Science* 38, 289-305.
16
17 Androstenone gives a urine or perspiration-like
18 odour, whilst indole and skatole give a camphor-like
19 odour. Levels of androstenone and skatole are each
20 increased in non-castrated boars, although the reason
21 for increased skatole levels has not been
22 established. Additionally the formation of
23 androstenone and skatole appears to be independent
24 although the degradation of these compounds is
25 currently believed to follow similar pathways and may
26 each involve cytochrome P450s. There remains debate
27 concerning the relative importance of androstenone
28 and skatole in contributing to boar taint, and in
29 certain studies emphasis has been placed onto
30 androstenone (see WO 98/41861 and WO 99/18192).
31 Methods that address the variation in levels of both

1 compounds would be particularly useful for breeding
2 male slaughter pigs.

3

4 Skatole (3-methyl-indole) is produced by the
5 breakdown of tryptophan by bacteria in the hindgut of
6 pigs and other animals (see Moss et al., "Boar taint:
7 the role of skatole", Meat Focus International,
8 October 1992; and Babol et al., "Boar taint in entire
9 male pigs", EAAP Publication No 92). Skatole is
10 absorbed into the bloodstream and through the portal
11 vein reaches the liver where it is metabolised. A
12 number of isoforms of P450 exist but literature, and
13 our own unpublished work suggest that metabolism of
14 skatole depends on the P4502E1 isoform (Babol, J.,
15 Squires, E.J. and Lundstrom, K. (1998) Hepatic
16 metabolism of skatole in pigs by cytochrome P4502E1
17 *J. Anim. Sci.* 76, 822-828 Squires, E.J. and
18 Lundstrom, K. (1997) Relationship between cytochrome
19 P4502E1 in liver and levels of skatole and its
20 metabolites in intact male pigs. *J. Anim. Sci.* 75,
21 2506 -2511).

22

23 Skatole that is not metabolised for some reason is
24 deposited in fatty tissues.

25

26 Methods for the identification and production of
27 swine with reduced boar taint are described in WO
28 99/18192. The method of WO 99/18192 is concerned
29 with androstenone production and in particular the
30 predicted impact of specific natural or
31 experimentally induced mutations or polymorphisms in

1 the porcine CYP17 gene that encodes cytochrome
2 P450c17. Cytochrome P450c17 is required for
3 production of androstenone. No experimental data are
4 provided to substantiate the claims - either of
5 naturally occurring CYP17 variants in pigs or of
6 experimentally induced mutations in the porcine CYP17
7 gene. A method for determining predisposition to
8 boar taint is disclosed in WO 98/41861. The method
9 of WO 98/41861 is concerned with assaying for the
10 presence of a low molecular weight isoform of
11 cytochrome b5. Cytochrome b5 is involved with
12 cytochrome P450c17 in the synthesis of androstenone.
13 Although data relating levels of cytochrome b5 to
14 levels of androstenone are presented, no evidence of
15 a genetic component of the differences is presented.
16 Neither the methods of WO 99/18192 nor WO 98/41861
17 address the contribution of skatole or indole.
18 Skatole is critical to consideration of 'boar taint'.
19 While about 25% of consumers are not able to smell
20 androstenone (Claus, 1978. *Der Geschlechtsgeruch des*
21 *Ebers aus der Sicht des Tierarztes, des Verbrauchers*
22 *und der Tierproduktion. Wien. Tierarztztl Mschr*
23 *65(12), 381-388) skatole is detected by all persons.*
24 Moreover, as skatole formation is not limited to the
25 boar, an understanding of skatole production and
26 clearance may be valuable in other meat species.
27
28 Previous research has suggested that part of the
29 variation in boar taint or its component traits may
30 be under genetic control.
31

1 Thus, Lundström and co-workers concluded from a study
2 of skatole levels in pig selection lines that there
3 is a genetic effect on skatole deposition which may
4 be due to a recessive allele of a major gene
5 (Lundström et al., 1994. Skatole levels in pigs
6 selected for high lean tissue growth rate on
7 different dietary protein levels. *Livest. Production*
8 *Science* 38, 125-132).

9

10 Genetic selection

11

12 Selection against animals with a genetic
13 predisposition to boar taint would be an attractive,
14 cost-effective and humane solution to the problem of
15 boar taint.

16

17 Skatole is believed to be the most important
18 component of boar taint; boar taint being observed
19 when not all skatole is degraded in the liver. A
20 genetic component of boar taint could therefore be
21 linked to a polymorphism in the cytochrome P450
22 isoform involved in skatole metabolism

23

24 We have now established that P4502E1 is the only P450
25 isoform involved in metabolism of skatole in the
26 liver. Moreover we have confirmed that in Large
27 White pigs, high liver levels of P4502E1 coincide
28 with low levels of skatole in the backfat and vice
29 versa. The mRNA levels of P4502E1 have been
30 demonstrated to exhibit a similar relationship with
31 skatole backfat levels.

1 Analysis has shown that animals with low skatole
2 levels have a cDNA sequence similar to the one
3 published by GenBank on the website
4 www.ncbi.nlm.nih.gov under the reference
5 Genbank/EMBL/DDBJ accession number: AB000885 (Kimura.
6 M, Suzuki, H. and Hamasima, N., 1999. Cloning of the
7 pig cytochrome P-450-j gene). However, the sequences
8 of high skatole Large Whites are different from those
9 of low skatole Large Whites in two locations. First,
10 at position 648 of the database sequence
11 Genbank/EMBL/DDBJ accession number: AB000885 low
12 skatole pigs have a C, while high skatole pigs have a
13 T; this does not change the amino acid sequence.
14 Second, at position 1435 the G observed in low
15 skatole pigs is changed to an A in high skatole pigs,
16 resulting in an alanine residue in the expressed
17 protein being changed to threonine. The
18 complementary polymorphisms were detected in the
19 complementary strand.

20

21 The experimental data herein presented provides
22 evidence that:

- 23 i. skatole levels are inversely related to
24 mRNA levels for P4502E1;
- 25 ii. polymorphisms exist at two locations in the
26 coding sequence for P4502E1 and that this
27 is associated with backfat skatole level;
- 28 iii. a polymorphism exists that changes the
29 amino acid composition of P4502E1 and this
30 is associated with backfat skatole levels;
31 and

1 iv. in Meishans*Large White crosses a different
2 factor than P4502E1 operates and causes
3 levels of skatole in backfat to be high.
4 Polymorphisms at location 648 and 1435
5 exist like in LW, but these are not
6 associated with skatole levels.

7
8 In one aspect, the present invention provides a
9 polynucleotide having a nucleotide sequence as set
10 out in SEQ ID No 1 or SEQ ID No 3, their
11 complementary sequences and the amino acid sequence
12 derived therefrom. Further the present invention
13 provides the use of these nucleotide sequences or
14 portions thereof for use as genetic markers in
15 screening pigs for boar taint phenotype. Preferably
16 the genetic markers will include nucleotides 645 to
17 650 or nucleotides 1432 to 1438.

18
19 In a further aspect, the present invention provides
20 an assay or method to identify pigs with a genetic
21 predisposition that reduces the incidence of boar
22 taint, wherein said assay comprises:
23 a) obtaining a DNA sample from a test pig;
24 b) analysing the sample to determine the allelic
25 variant(s) at position 648 or 1435 or both;
26 c) using said results to select for animals of the
27 preferred genotype.

28
29 The polymorphisms were found within the coding
30 sequence of P4502E1 and the 1435 polymorphism
31 actually alters the amino acid composition of the

10

1 expressed protein, and it is believed that one or
2 both polymorphisms are responsible for boar taint
3 phenotype via alterations to the function or
4 expression of P4502E1. However, this has not been
5 conclusively established and it remains possible that
6 the polymorphisms described above (either separately
7 or together) do not affect the function or expression
8 of P4502E1 itself, but may be linked to the actual
9 causative mutation elsewhere in the genome. In the
10 latter case, the polymorphisms described herein will
11 act as genetic markers. It is known to those skilled
12 in the art that other genetic markers with a similar
13 linkage may exist in the same region of the genome
14 and they can be used instead. Linkage of these other
15 genetic markers with skatole levels is part of the
16 present invention.

17

18 Thus, the present invention provides a method to
19 identify pigs with a genetic predisposition to a
20 reduced incidence of boar taint, wherein said method
21 comprises:

22

- 23 a) obtaining DNA samples from a population of
- 24 pigs;
- 25 b) genotyping at least a sample of said population
- 26 for at least one of (preferably both of) the
- 27 polymorphism(s) described above;
- 28 c) measuring boar taint traits for at least a
- 29 sample of said population;
- 30 d) correlating the presence of allelic variants of
- 31 said polymorphism(s) with said traits;

11

- 1 e) obtaining a DNA sample from a test pig;
- 2 f) analysing the sample to determine the allelic
- 3 variant(s) present at a said polymorphism; and
- 4 g) using the results obtained to select for
- 5 animals of the preferred genotype.

6

7 The invention further relates to a method to
8 approximate the actual boar taint level of a test pig
9 wherein the method comprises:

- 10 a) obtaining a DNA sample from a test pig;
- 11 b) analysing the sample to determine the
- 12 allelic variant at position 648 or 1435 or
- 13 both;
- 14 c) using said results to approximate skatole
- 15 levels in said test pig.

16

17 Preferably this method comprises:

- 18 a) obtaining DNA samples from a population
- 19 of pigs;
- 20 b) genotyping at least a sample of said
- 21 population for at least one (preferably
- 22 both of) the polymorphism(s) occurring at
- 23 positions 648 and 1435 of the P4502E1
- 24 coding sequence;
- 25 c) measuring boar taint traits for at least
- 26 a sample of said population;
- 27 d) correlating the presence of allelic
- 28 variants of said polymorphism(s) with
- 29 said traits;
- 30 e) obtaining a DNA sample from a test pig;

12

- 1 f) analysing the sample to determine the
2 allelic variant(s) present at a said
3 polymorphism; and
4 g) using the results obtained to approximate
5 skatole levels in said test pig.

6

7 Preferably the polymorphism is the allelic variant at
8 position 648 or 1435 of the coding sequence for
9 P4502E1 or a combination of the two. Other genetic
10 markers that map within or close to P4502E1 may also
11 be used, preferably in addition to the polymorphisms
12 referred to above.

13

14 The animals shown to have marker genotypes or
15 predicted genotypes indicative of a desirable boar
16 taint predisposition (for example boars identified to
17 have reduced boar taint), or the close relatives of
18 such animals, can be used in a breeding program, as
19 breeding stock or for meat production.

20

21 In the assay or method of the present invention, the
22 genomic DNA will be detected from a sample of porcine
23 origin but the exact tissue forming the sample is not
24 limited as long as it contains genomic DNA. Examples
25 include body fluids such as blood, sperm, ascites and
26 urine, tissue cells such as liver tissue, muscle,
27 skin, hair follicles, fat and testicular tissue. The
28 genomic DNA to be analysed can be prepared by
29 extracting and purifying the DNA from such samples.

30

1 The method may be conducted *in vitro* or *in vivo* using
2 a sample from a living animal or *post mortem*
3 following the death of the animal being tested. If
4 the assay is conducted *post mortem*, the information
5 obtained may be of use for the siblings, parents or
6 other close relatives of the animal.

7
8 Any suitable method may be used to determine the
9 nucleotides at positions 648 and/or 1435. Mention
10 may be made of the following suitable methods
11 (although other methodologies may also be used).
12 PCR-RFLP (polymerase chain reaction - restriction
13 fragment length polymorphism), OLA (oligonucleotide
14 ligation amplification), and methods for detecting
15 single nucleotide polymorphisms (SNPs) including, but
16 not limited to, hybridization-based methods, Third
17 Wave's Invader technology and mass spectrometry-based
18 methods.

19
20 Either of the polymorphisms in P4502E1 disclosed
21 herein may prove to be the functional mutation or
22 alternatively allow the isolation and
23 characterisation of the functional mutation itself.

24
25 It remains possible that P4502E1 is not itself
26 responsible for the observed variation in skatole
27 levels, but merely contains a genetic marker linked
28 with the functional mutation. Nonetheless (since the
29 positioning of the mutation enables a search for
30 linkage to the genes responsible for the trait) the
31 present finding will facilitate identification of the

1 functional mutation. Once this mutation is located
2 the option to manipulate the trait genes by
3 transgenesis or to develop a further assay or method
4 arises and forms part of the present invention.

5

6 The present invention will now be described in more
7 detail by reference to the following, non-limiting,
8 examples and figures in which:

9

10 Fig. 1 shows the rate of skatole metabolism in
11 isolated liver microsomes as a function of microsome
12 P4502E1 content confirming that the rate of skatole
13 metabolism depends only on the content of P4502E1.
14 Microsomes were isolated from seven different pigs
15 and the P4502E1 content of each preparation was
16 determined. A linear relationship was obtained ($y =$
17 $0.034 (x) + 0.004$; correlation coefficient = 0.92).

18

19 Fig. 2a shows inhibition of microsomal skatole
20 metabolism by allyl sulfide- a specific inhibitor of
21 cytochrome P4502E1. Allyl sulphate was added to the
22 incubation at a concentration of 1mM at zero time.
23 Each point represents the mean \pm S.E.M. for three
24 independent experiments.

25

26 Fig. 2b shows that another specific inhibitor,
27 chlorzoxazone (0.025 - 0.2 M), progressively
28 inhibited metabolism of skatole, when measured after
29 40 minutes incubation.

30

1 Fig. 3 shows the relationship between backfat skatole
2 and P4502E1 levels in liver microsomes. Microsomes
3 were isolated from the livers of 12 Large White and 8
4 Meishan*Large White crosses pigs exhibiting a wide
5 range of adipose tissue skatole levels. The results
6 are from a number of different blots and the P4502E1
7 levels are normalised to 100% for one specific
8 microsomal preparation, which was included on each
9 blot.

10

11 Fig. 4 shows the correlation between hepatic P4502E1
12 mRNA levels and adipose tissue skatole content. RNA
13 was extracted from liver samples from a number of
14 pigs with different adipose tissue skatole levels and
15 probed with P4502E1-specific DNA. The results are
16 derived from several blots and are normalised to 100%
17 for one RNA sample, which was present on all blots.

18 Fig. 5 shows a part of the cDNA sequence coding for
19 P4502E1 in pigs for one pig with high skatole and one
20 with low skatole.

21

22 Fig. 6 shows the complete cDNA sequence (SEQ ID No 1)
23 coding for P4502E1 for pigs with low skatole compared
24 to the cDNA sequence Genbank/EMBL/DDBJ accession
25 number: AB000885 published by GenBank.

26

27 Fig. 7 shows the amino acid sequence (SEQ ID No 2)
28 derived from SEQ ID No 1 for pig with low skatole
29 compared to the amino acid sequence coded by the cDNA
30 sequence Genbank/EMBL/DDBJ accession number: AB000885
31 shown in Fig. 6.

16

1 Fig. 8 shows the cDNA sequence (SEQ ID No 3) coding
2 for P4502E1 for one pig with high skatole.

3

4 Fig. 9 shows part of the amino acid sequence (SEQ ID
5 No 4) derived from SEQ ID No 3 for pig with high
6 skatole compared to amino acid sequence coded by the
7 cDNA sequence Genbank/EMBL/DDBJ accession number:
8 AB000885 shown in Fig. 8.

9

10 Fig. 10 shows part of the amino acid sequence (SEQ ID
11 No 5) derived from SEQ ID No 3 for pig with high
12 skatole compared to amino acid sequence coded by the
13 cDNA sequence Genbank/EMBL/DDBJ accession number:
14 AB000885 shown in Fig. 8.

15

16 Example 1

17

18 Measurement of skatole metabolism by thin layer 19 chromatography

20

21 Samples of liver were obtained at 15 minutes post-
22 mortem from intact male Large White pigs, frozen
23 immediately in solid CO₂ and subsequently stored at -
24 80°C for up to 2 months. Microsomes were isolated as
25 described by Schenkman and Cinti, 1978 (Preparation
26 of microsomes with calcium. *Methods Enzymol.* 52, 83-
27 89). A sample of pig liver (10 g) was homogenised
28 with a "Polytron" homogeniser (Kinematica,
29 Switzerland) for 1 minute in 40 ml of sucrose buffer
30 (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4), followed by
31 centrifugation at 3,000 x g and 12,000 x g in order

1 to obtain the post-mitochondrial supernatant. Solid
2 CaCl_2 was added at a final concentration of 8 mM and
3 microsomes were sedimented at 25,000 x g for 15
4 minutes. The microsomal fraction was washed with KCl
5 buffer (150 mM KCl, 10 mM Tris-HCl, pH 7.4). The
6 pellet was suspended at a protein concentration of
7 about 20 mg/ml in a medium containing 50 mM Tris-HCl,
8 10 mM KH_2PO_4 , 0.1 mM EDTA, 20% glycerol and
9 inhibitors of proteolytic enzymes (0.1 mM
10 phenylmethylsulfonyl fluoride and 1 μg per ml
11 pepstatin + antipain + leupeptin). Isolated
12 microsomes were stored in liquid nitrogen for up to
13 month. The rates of skatole metabolism in microsomes
14 prepared from fresh and frozen samples of the same
15 liver were found to be identical. The P4502E1
16 protein level in isolated microsomes was determined
17 by Western Blotting using a commercial antibody.
18 Briefly microsomal proteins were separated by SDS-
19 PAGE (Cleveland, Fischer, Kirschner, Laemmli, 1977.
20 Peptide mapping by limited proteolysis in sodium
21 dodecyl sulfate and analysis by gel electrophoresis.
22 *J.Biol.Chem.* 252, 1102-1106) and electro-blotted on
23 to nitrocellulose. After successive incubations with
24 anti-P4502E1 and peroxidase-labelled anti-rabbit IgG
25 the blot was developed using an ECL procedure. The
26 film was scanned and the 55kD bands were quantified
27 using Imagequant programme (Molecular Dynamics).
28 Isolated pig liver microsomes were incubated in 100
29 μl (total volume) of the medium, containing 50 mM
30 Tris-HCl, 10 mM K_2HPO_4 , 0.1 mM EDTA, 20% glycerol, pH
31 7.4 at 37°C in the presence of various concentrations

18

1 of skatole together with 1mM NADH plus 1mM NADPH as
2 cofactors. The reaction was stopped by addition of
3 100 µl of ice-cold methanol. A zero time control was
4 performed by adding methanol simultaneously with the
5 microsomes. The mixture was vortexed for 1 minute
6 and centrifuged for 10 minutes at 2,000 x g to
7 precipitate the protein. The supernatant containing
8 skatole was used for the skatole assays.

9

10 After centrifugation the supernatant was applied to a
11 TLC plate in hexane:ether (4:1) and the plate was
12 stained with Ehrlich reagent. The corresponding
13 amount of pure skatole was run simultaneously. In
14 such incubations two spots were obtained - a purple
15 spot representing skatole and a pink spot at the
16 origin representing any skatole metabolites which
17 react with Ehrlich reagent. This latter spot was
18 absent at zero time. During the progress of the
19 reaction the intensity of the skatole spots decreased
20 and that of the spots at the origin increased.
21 Metabolites of skatole are not commercially available
22 and therefore cannot be used as internal standards.
23 Further, not all skatole metabolites react with
24 Ehrlich reagent. Therefore in order to confirm that
25 skatole was completely separated from its products by
26 this procedure, samples from the same incubations
27 were run on TLC and were also analysed by HRGC which
28 allows complete separation and detection of all the
29 skatole metabolites in the mixture.

30

1 Protein was determined by the Bradford method
2 (Bradford, 1976. A rapid and sensitive method for the
3 quantitation of microgram quantities of protein
4 utilising the principle of protein-dye binding. *Anal*
5 *Biochem* 7;72:248-254) using bovine serum albumin as a
6 standard.

7
8 Figure 1 shows skatole metabolism by isolated pig
9 liver microsomes in the presence of oxygen, NADH and
10 NADPH. Measurements of skatole by the Thin Layer
11 Chromatography (TLC) method coincided closely with
12 High Resolution Gas Chromatography (HRGC) measurement
13 of the same samples, thus validating the method.

14

15 Example 2

16 Determination of the P450 isoform involved in skatole 17 metabolism.

18

19 The above experimental system described in Example 1
20 was used to investigate the involvement of cytochrome
21 P4502E1 in skatole metabolism by pig liver
22 microsomes. In initial experiments SKF-525A (0.1
23 mM), a general P450 inhibitor, completely inhibited
24 skatole disappearance when measured over 15 minutes
25 indicating that all skatole metabolism via the
26 cytochrome P450 system (data not shown). Figure 2a
27 shows that allyl sulphate, a specific P4502E1
28 inhibitor, completely inhibited skatole metabolism
29 when added at over a 60 minute incubation period.
30 Figure 2b shows that another specific inhibitor,
31 chlorzoxazone (0.025 - 0.2 M), progressively

1 inhibited metabolism of skatole, when measured after
2 40 minutes incubation.

3

4 These results indicate that, in agreement with
5 previous findings in the literature, skatole is
6 metabolised via P4502E1.

7

8 Example 3

9

10 Relationship between P4502E1 content, backfat 11 skatole and rate of microsomal skatole metabolism.

12

13 Liver samples were frozen in solid CO₂ within minutes
14 of slaughter and kept at -80°C. Microsomes were
15 isolated from frozen livers of selected pigs with
16 various backfat skatole levels. Levels of P450 in
17 the microsomes were derived from Western Blotting
18 experiments using a commercial antibody stated to be
19 specific for P4502E1. The initial rate of skatole
20 metabolism was measured in the same preparations.
21 Initial experiments showed that the rate of
22 metabolism in microsomes from fresh liver was the
23 same as those from liver frozen at -80°C for some
24 weeks. Figure 3 shows that pigs with low backfat
25 skatole levels all had high levels of P4502E1. Some
26 pigs with high skatole backfat levels had low levels
27 of P4502E1, but in a number of Meishan*Large White
28 crosses pigs the P4502E1 level was only marginally
29 reduced. In Large White pigs, the findings are
30 similar to those of others (Squires and Lundstrom,
31 1997. Relationship between cytochrome P4502E1 in

21

1 liver and levels of skatole and its metabolites in
2 intact male pigs. *J. Anim. Sci.* 75, 2506 -2511). In
3 Meishan*Large White crosses pigs a different
4 mechanism operates by which high skatole levels can
5 exist with high P4502E1 levels.

6 However, the rate of skatole metabolism in microsomes
7 varied by less than a factor of 1.5 when the P4502E1
8 level varied by a factor of 10 (Figure 4). This is
9 consistent with the previous results of Babol,
10 Squires and Lundstrom, 1998 (Relationship between
11 oxidation and conjugation metabolism of skatole in
12 pig liver and concentration of skatole in fat *J.*
13 *Anim. Sci.* 76, 829-838). There was no correlation
14 between rates of microsomal skatole metabolism and
15 backfat skatole in the samples measured.

16

17 **Example 4**

18

19 Determination of P4502E1 mRNA in liver by Northern 20 blotting

21

22 A 375bp cDNA probe corresponding to bases 507 - 881
23 of the pig P4502E1 sequence Genbank/EMBL/DDBJ
24 accession numbers: AB000885 was generated by
25 Polymerase chain reaction (PCR) with pig liver cDNA
26 as template; the identity of the probe was checked by
27 DNA sequencing. The probe was labelled with α -³²P
28 dCTP using the Boehringer Hi-Prime kit. Total RNA
29 was extracted from frozen liver using Tri-Reagent
30 (Sigma) and 20 μ g RNA was separated on an agarose gel

1 as described by Maniatis et al., Sambrook et al. (in
2 Sambrook, Fritsch, Maniatis, 1989. Analysis of RNA.
3 *Molecular cloning*. A laboratory manual, 1, 7.37-
4 7.57). After pre-hybridisation the blot was
5 hybridised overnight at 42°C and washed at 42°C in
6 SSPE followed by 2 washes in 50°C in SSPE/SDS 0.1%.
7 After autoradiography, the bands were quantified by
8 scanning using the Imagequant programme.

9
10 Figure 4 shows that there was an inverse correlation
11 between backfat skatole and P4502E1 mRNA expression
12 in Large White pigs. This confirms that P4502E1 is
13 involved in skatole metabolism.

14

15 Example 5

16

17 DNA sequencing

18

19 We have used three Large White pigs with very low
20 skatole (0.019, 0.026 and 0.118 µg/mg backfat), three
21 Large White pigs with high skatole (1.309, 0.740 and
22 0.400 µg/mg backfat) and one Meishan*Large White
23 crosses pig with high skatole (0.914 µg/mg backfat).

24

25 RNA was isolated from each liver using Tri-Reagent.
26 First strand DNA was synthesised using reverse
27 transcriptase and oligo dT priming. PCR primers were
28 designed corresponding to various locations on the
29 database sequence of pig P4502E1. The DNA was used
30 as a template for PCR. The single PCR product of the

1 correct size was extracted, ligated into the pGem
2 vector and used to transform E. coli (XL-1Blue). The
3 insert size was checked after double digestion with
4 EcoRI and the insert was sequenced in the plasmid
5 using M13 forward and reverse primers. The primers
6 used were:

7

8 Forward primer: 5' CATCTCCATCTGGAAGCACATC 3'

9 Reserve primer: 5' ACACTTGTGAGCGGGAATG 3'

10

11 Figure 5 shows cDNA sequences for the five animals
12 and the corresponding sequence on the database
13 Genbank/EMBL/DDBJ accession number: AB 000885 showing
14 differences between the sequence entry
15 (Genbank/EMBL/DDBJ accession number: AB000885) and
16 low skatole pigs on one hand and high skatole pigs on
17 the other at locations 648 and 1435.

18

19 Additionally, differences have been found between the
20 coding sequence of all seven pigs and the sequence
21 Genbank/EMBL/DDBJ accession number: AB00085. In
22 addition to the differences appearing at the
23 extremities of the cDNA sequence, two differences in
24 the coding region at positions 1087 and 1180-1181
25 have been found.

26

27 Studies carried on two additional high skatole
28 Meishan*Large White crosses pigs have shown that
29 amongst the three high skatole pigs analysed two have
30 the same polymorphisms seen in the high skatole Large
31 White pigs while the other does not. These findings

24

1 are in line with the fact that we find that in
2 Meishan*Large White crosses different or additional
3 mechanisms may operate.

4

5 **Example 6**

6

7 PCR-RFLP assay for polymorphisms at nt 1435

8

9 A PCR-RFLP assay for the polymorphism at the
10 nucleotide (nt) corresponding to nt 1435 in the cDNA
11 sequence has been developed. The sequence CGCG at nt
12 1434-1437 in the cDNA sequence corresponds to the
13 cleavage site for the restriction endonuclease BstUI.
14 When this sequence is CACG the sequence is not
15 recognised or cleaved by BstUI. We predicted the
16 location of the exon-intron boundaries in the cDNA
17 sequence by comparison with the human *CYP2E* gene
18 (EMBL/Genbank accession number: J02843).
19 Oligonucleotide primers with the following sequences
20 were designed for the amplification of a 172 bp
21 fragment including the polymorphic nucleotide from
22 genomic DNA.

23

24 Forward primer: 5'-GGGTGTGTGTCGGAGAGG-3'

25 Reverse primer: 5'-CGGGGAATGACACAGAGTTT-3'

26

27 Amplification of the 172bp fragment from genomic DNA
28 was effected by the Polymerase Chain Reaction (PCR)
29 in a total volume of 50 microlitres. PCR reactions
30 contained 100 ng genomic DNA, 1 x PCR buffer (Roche),
31 1.5 mM MgCl₂, 100 µM dNTPs, 500 nM each primer, 1

1 unit Taq DNA polymerase. PCR conditions were 94°C
for 3mins, then 35 cycles of 94°C for 30 seconds,
56°C for 45 seconds and 72°C for 1 min. PCR products
were digested by adding 10 units of BstUI to the
reaction mix and incubating at 60°C overnight. The
digested PCR products were fractionated by
electrophoresis through a 2.5% Metaphor™(Flowgen)
agarose gel. Where nt 1435 is a cytosine (C) the 172
bp fragment is cleaved by BstUI to yield products of
142 and 30 bps. Where nt 1435 is an adenine (A) the
172 bp fragment is not cleaved by BstUI. In samples
from animals with one C and one A allele (i.e.
heterozygotes) fragments of 172, 142 and 30 bp are
observed.

1 CLAIMS

2

3 1. A polynucleotide having a nucleotide sequence
4 as set out in SEQ ID No 1 or 3 or a
5 complementary sequence thereof.

6

7 2. An assay to identify pigs with a genetic
8 predisposition to boar taint wherein said assay
9 comprises:

- 10 a) obtaining a DNA sample from a test pig;
11 b) analysing the sample to determine the
12 allelic variant at position 648 or 1435
13 or both;
14 c) using said results to select for animals
15 of the preferred genotype.

16

17 3. The assay as claimed in Claim 2 wherein the
18 allelic variation at position 648 is
19 determined.

20

21 4. The assay as claimed in Claim 2 wherein the
22 allelic variation at position 1435 is
23 determined.

24

25 5. The assay as claimed in Claim 2 wherein other
26 genetic markers that map within or close to
27 P4502E1 are used in addition to or instead of
28 determining the allelic variant at position 648
29 or 1435 or both.

30

31 6. The assay as claimed in any one of Claims 2 to
32 5 wherein the nucleotides at positions 648 or

- 1 1145 are determined by PCR-RFLP, OLA, mass
2 spectrometry based methods, methods for
3 detecting single nucleotide polymorphisms
4 (SNPs) like hybridization-based methods, or
5 Third Wave's Invader technology.
6
- 7 7. The assay as claimed in any one of Claims 1 to
8 6 wherein the method is conducted on the test
9 pig post mortem.
10
- 11 8. A method to identify pigs with a genetic
12 predisposition to boar taint comprising:
13 a) obtaining a DNA sample from a test
14 pig;
15 b) analysing the sample to determine
16 the allelic variant at position 648
17 or 1435 or both;
18 c) using said results to select for
19 animals of the preferred genotype.
20
- 21 9. A method to identify pigs with a genetic
22 predisposition to a reduced incidence of boar
23 taint, wherein said method comprises:
24
- 25 a) obtaining DNA samples from a population
26 of pigs;
27 b) genotyping at least a sample of said
28 population for at least one (preferably
29 both of) the polymorphism(s) occurring at
30 positions 648 and 1435 of the P4502E1
31 coding sequence;

- 1 c) measuring boar taint traits for at least
- 2 a sample of said population;
- 3 d) correlating the presence of allelic
- 4 variants of said polymorphism(s) with
- 5 said traits;
- 6 e) obtaining a DNA sample from a test pig;
- 7 f) analysing the sample to determine the
- 8 allelic variant(s) present at a said
- 9 polymorphism; and
- 10 g) using the results obtained to select for
- 11 animals of the preferred genotype.
- 12
- 13 10. A method as claimed in Claim 9 wherein other
- 14 genetic markers that map within or close to
- 15 P4502E1 are used in addition to or instead of
- 16 the polymorphisms of step b).
- 17
- 18 11. A method of selecting an animal for use in a
- 19 breeding program, said method comprising using
- 20 said animal as a test animal in step e) of
- 21 Claim 9.
- 22
- 23 12. A method as claimed in Claim 9 wherein other
- 24 genetic markers that map within or close to
- 25 P4502E1 are used in addition to or instead of
- 26 the polymorphisms occurring at positions 648
- 27 and 1435 of the P4502E1 coding sequence.
- 28
- 29 13. A method to approximate the actual boar taint
- 30 level of a test pig wherein said method
- 31 comprises:
- 32 a) obtaining a DNA sample from a test pig;

29

- 1 b) analysing the sample to determine the
2 allelic variant at position 648 or 1435 or
3 both;
4 c) using said results to approximate skatole
5 levels in said test pig.

6

7 14. A method to approximate the actual boar taint
8 level of a test pig, wherein said method
9 comprises:

- 10 a) obtaining DNA samples from a population
11 of pigs;
12 b) genotyping at least a sample of said
13 population for at least one (preferably
14 both of) the polymorphism(s) occurring at
15 positions 648 and 1435 of the P4502E1
16 coding sequence;
17 c) measuring boar taint traits for at least
18 a sample of said population;
19 d) correlating the presence of allelic
20 variants of said polymorphism(s) with
21 said traits;
22 e) obtaining a DNA sample from a test pig;
23 f) analysing the sample to determine the
24 allelic variant(s) present at a said
25 polymorphism; and
26 g) using the results obtained to approximate
27 skatole levels in said test pig.

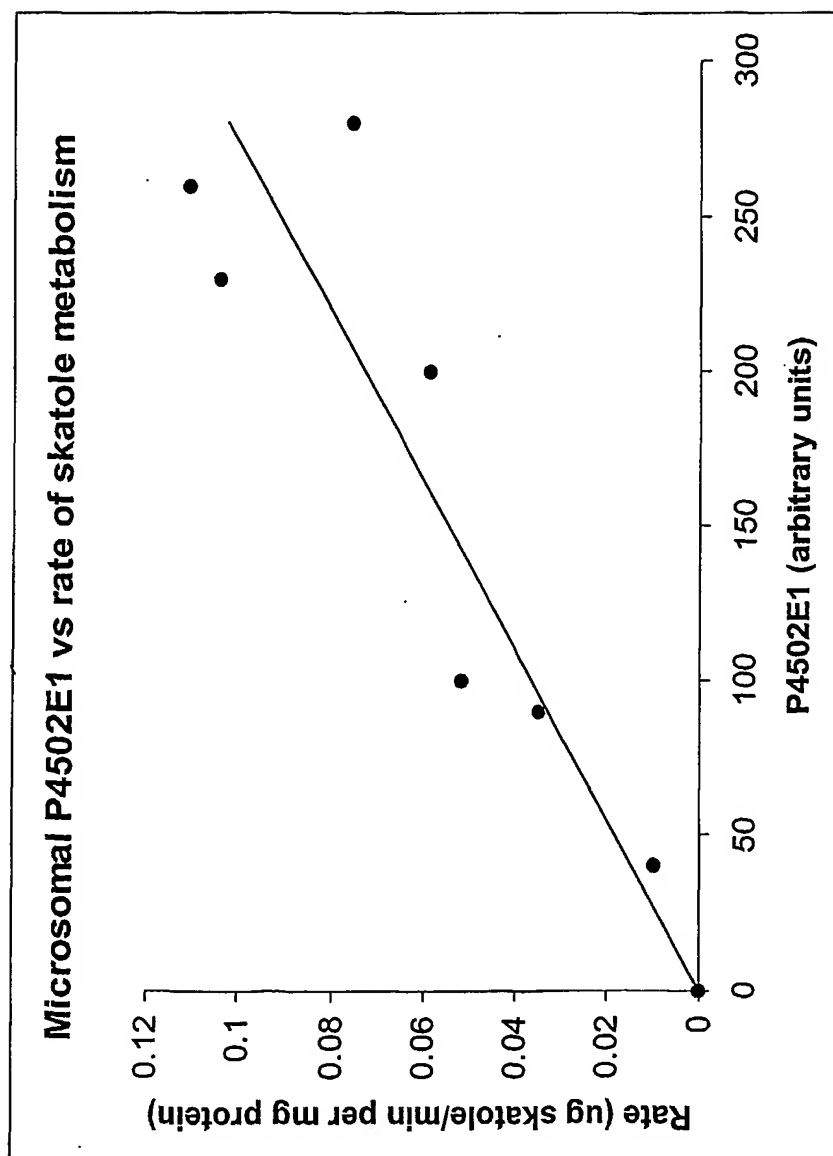
28

29 15. The method as claimed in any one of Claims 8 to
30 14 wherein the nucleotides at positions 648 or
31 1145 are determined by PCR-RFLP, OLA, mass
32 spectrometry based methods, methods for

1 detecting single nucleotide polymorphisms
2 (SNPs) like hybridization-based methods, or
3 Third Wave's Invader technology.
4

5 16. The method as claimed in any one of Claims 8
6 to 15 wherein the method is conducted on the
7 test pig post mortem.
8

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*Fig. 1*

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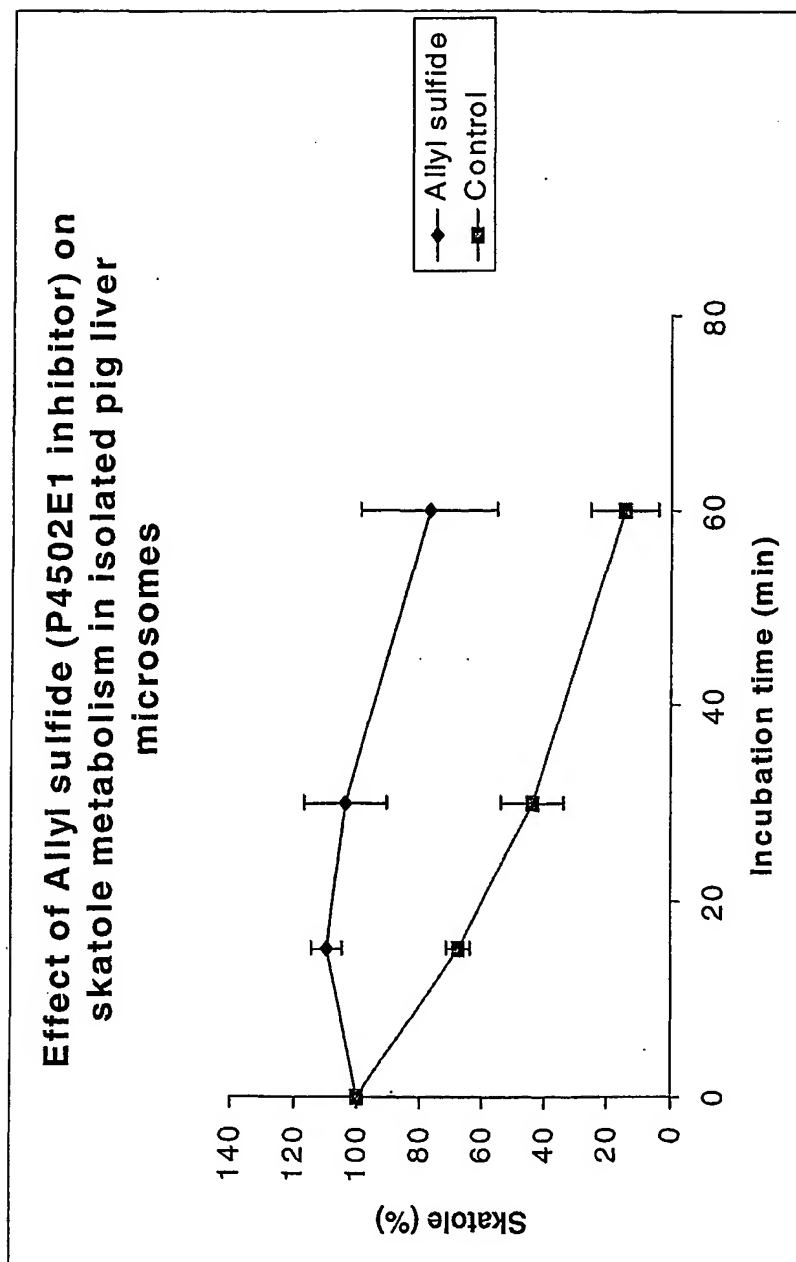


Fig. 2a

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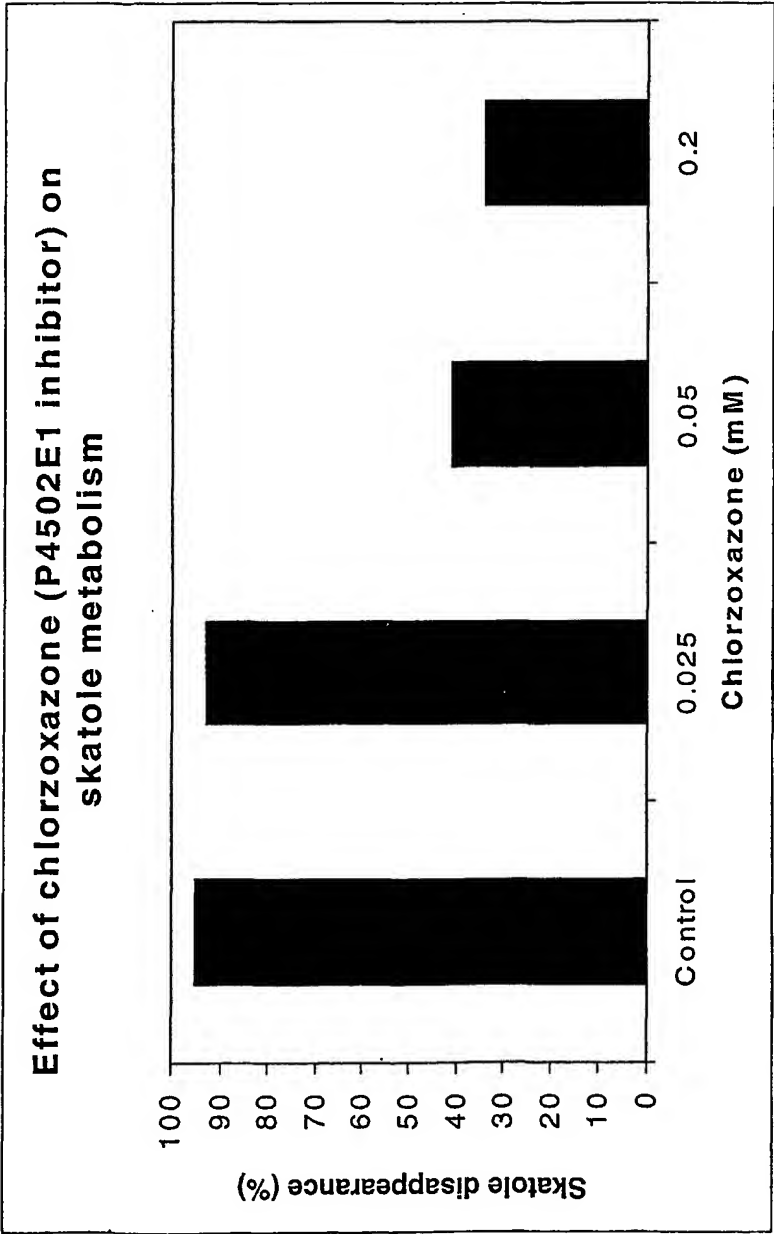


Fig. 2b

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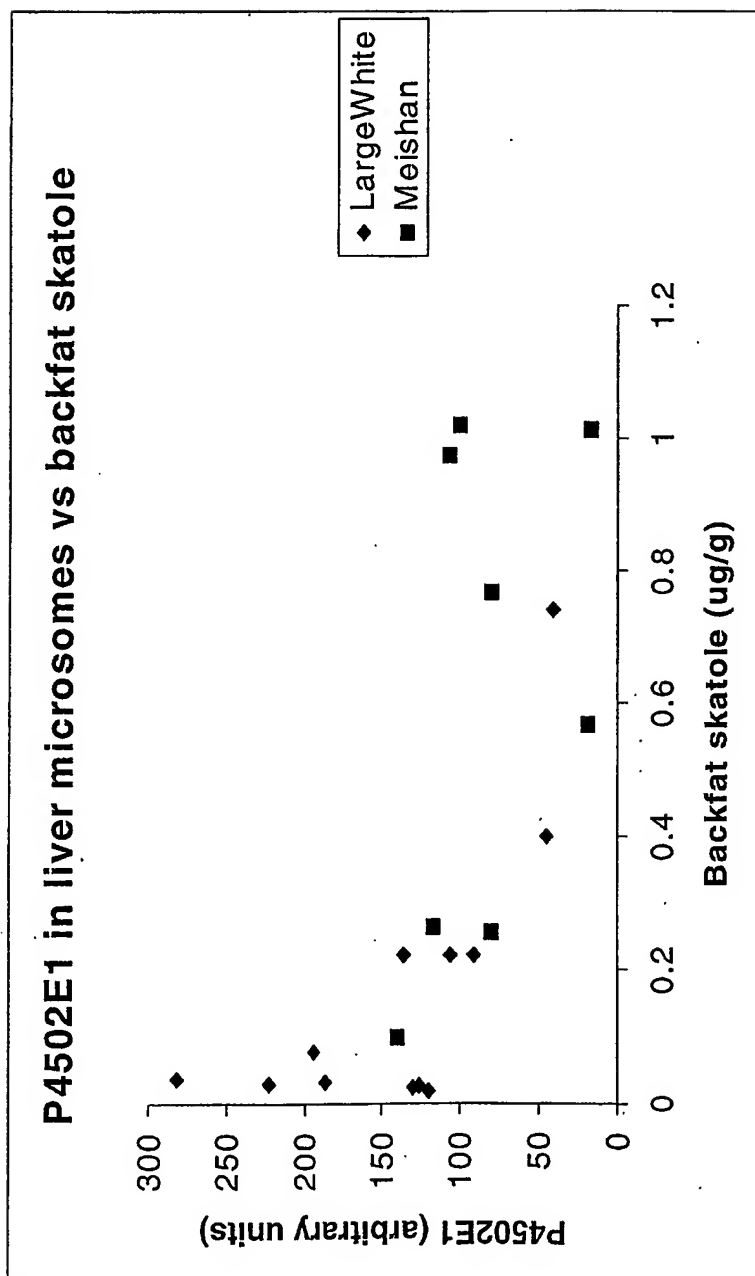


Fig. 3

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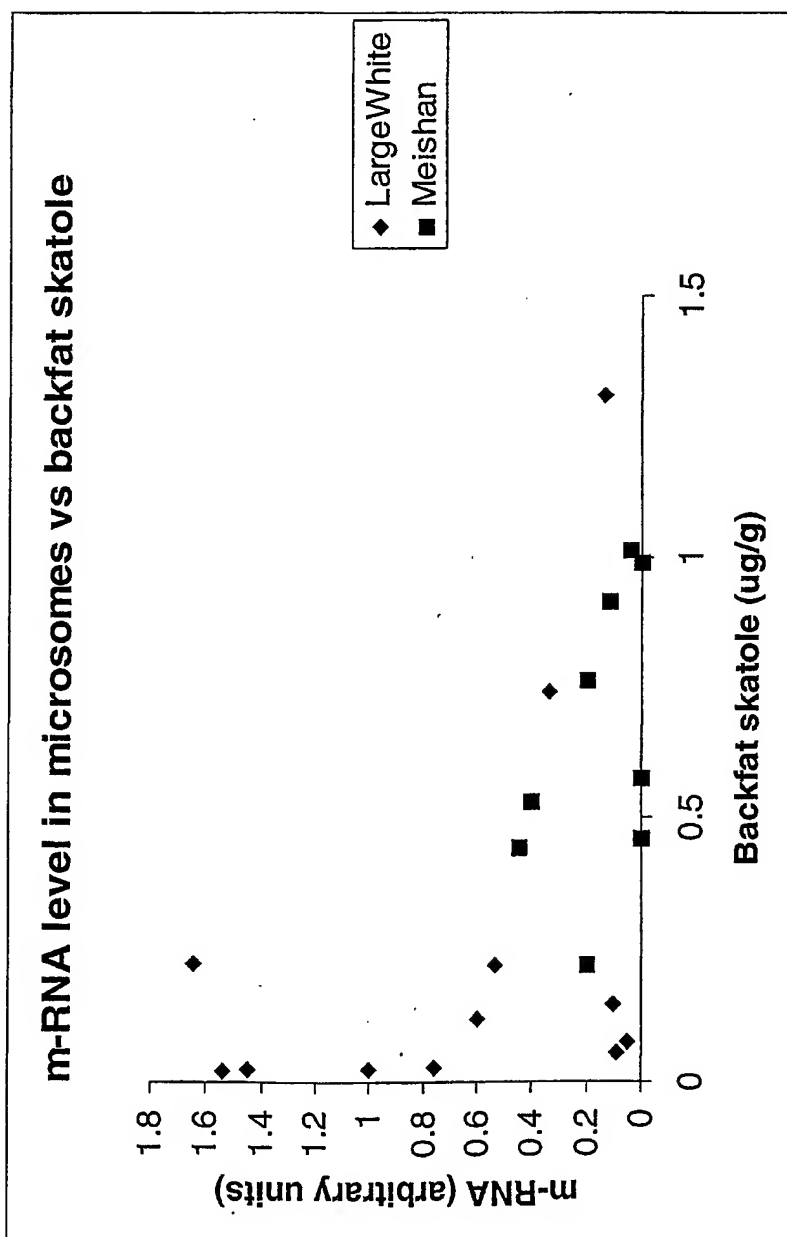


Fig. 4

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Partial sequence alignment of P4502E1 from Large White high skatole (1.309µg/g) pig (capitals) v. Large White low skatole (0.019µg/g) pig (small letters). Note polymorphisms at 648 and 1435.

```

TCTCCGACATCCTCTTCCGCCAGCACTTTGACTACAATGACAAGACCTGT
|||||
551 tctccgacatcctcttccgccagcactttgactacaatgacaagacctgt 600

CTGAGGTTAATGAGCATGTTCAATGAGAACTTCTACCTGCTCAGTACTGG
|||||
601 ctgaggttaatgagcatgttcaatgagaacttctacctgctcagtaccgg 650

CTGGATCCAGCTTTATAATAATTTCTCAGGCTATCTACGCTACCTGCCTG
|||||
651 ctggatccagctttataataatttctcaggctatctacgctacctgcctg 700

GAAGCCATAGGAACTAATGAAAAATATATCTGAAATAAAAGATTATGCT
|||||
701 gaagccataggaactaatgaaaaatataatctgaaataaaagattatgct 750

.....
CTGTTCCCTGTTTCATGGCTGCCATCTTGCAGCACTTTAACTTGAAATCTCT
|||||
1351 ctggttccctgttcatggctgccatcttgcagcactttaacttgaaatctct
1400

TGTTGACCCCAAGGATATCGACCTCAGCCCCATCAGGATTGGGTTTGCCA
|||||
1401 tgttgaccccaaggatatcgacctcagcccatcgcgattggggttgcca
1450

AGATTCACCCCATTAACAACCTCTGTGTCATTCCCGCTCACAAGTGT
|||||
1451 agattccccccattacaaactctgtgtcattccccgctcacaagtgt 1500

```

Fig. 5

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1 GCACACATTGAAAGATCCCCGGAAGGAGCC 50
 1cggcacgaggcc 12
 51 ATGACTGCCCTGGGCATCACGGTGGCCCTGCTGGTGTGGTGGTCACCCCT 100
 13 atgactgccctgggcatcacgggtggccctgctgggtgtggttggtcaccct 62
 101 GCTGCTCATCTCCATCTGGAAGCACATCCACAGTAGCTGGAACTTCCCC 150
 63 gctgctcatctccatctggaagcacatccacagtagctggaacttcccc 112
 151 CTGGCCCTTTCCCACTGCCCATCGTTGGGAACATTTTCAGTTGGACCTT 200
 113 ctggccctttcccactgcccatcgttgggaacattttccagttggacctt 162
 201 AAGAAATATCCCAATCCTTCACCATGCTGGCAGAGCGTTACGGGCCGGT 250
 163 aagaatattcccaatccttcacatgctggcagagcggtacgggcccgt 212
 251 GTTCACTGTGTACCTGGGTTCCGGCGCATTTGTGGTCTGACGGCTACA 300
 213 gttcaactgtgtacctgggttcgcgccgcatgtgtgctcctgcacggctaca 262
 301 AGGCGGTGAAGGAGGTCTTGCTCCACTACAAGAATGAGTTCTCTGGCAGA 350
 263 aggcgtgaaggaggtcttgctccactacaagaatgagttctctggcaga 312
 351 GGGGAAATCCCACGTTCCAAAGTGACACAGGACAAAGGGGTCAATTTCAA 400
 313 ggggaaatcccacgttccaagtgcacaaggacaaaggggtatatttcaa 362
 401 TAATGGACCAACCTGGCGGGACACTCGGCGGTCTCCCTCACCAACCTCC 450
 363 taatggaccaacctggcgggacactcggcggttctccctcaccacccctcc 412
 451 GTGACTTCGGGATGGGGAACAGGGCAATGAGCAGCGGATCCAGAGGGAG 500
 413 gtgacttcgggatggggaacagggcaatgagcagcggtccagagggag 462
 501 GCCCACTTCCTGCTGGAGGCACTCAGGAAGACCCATGGCCAGCCCTTGA 550
 463 gcccacttctctgctggaggcactcaggaagaccatggccagcccttga 512
 551 TCCCACTTCCTCATCGGCTGCGCACCTGCAATGTCATCTCCGACATCC 600
 513 tcccacttctctcatcggtgcgcaccctgcaatgtcatctccgacatcc 562
 601 TCTTCGCGCAGCACTTTGACTACAATGACAAGACCTGTCTGAGGTTAATG 650
 563 tcttcgcgacgactttgactacaatgacaagacctgtctgaggttaatg 612
 al.pair (40%)

SEQ ID No. 1

- AB000885

Fig. 6

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```

651 AGCATGTTCAATGAGAACTTCTACCTGCTCAGTAACGGCTGGATCCAGCT 700
|||||
613 agcatgttcaatgagaactttctacctgctcagtaacggctggatccagct 662
|||||
701 TTATAATAATTTCTCAGGCTATCTACGCTACCTGCCTGGAGCCATAGGA 750
|||||
663 ttataataatttctcaggctatctacgctacctgcctggaagccatagga 712
|||||
751 AACTAATGAAAAATATATCTGAAATAAAAGATTATGCTTTAGAAAGAGTG 800
|||||
713 aactaatgaaaaatataatgtgaaataaaagattatgctttagaagagtg 762
|||||
801 AAGGACCACCGGATTCACTGGAGCCGAGCTGTCCTCGAGATTCACTGA 850
|||||
763 aaggaccacccggattcactggagcccagctgtcctcgagattcactga 812
|||||
851 CACCTGCTGATGGAAATGGAGAAGGAAAAATACAGTGCAGAACCTATAT 900
|||||
813 caccctgctgatggaaatggagaaggaaaaatacagtgagaaacctatat 862
|||||
901 ACACCTTGGACAACATTGCCGTGACCGTGGCCGACATGTTCTTTGCGGGG 950
|||||
863 acaccttggacaacattgccgtgaccgtggccgacatgttctttgogggg 912
|||||
951 ACAGAGACCACAGCACCCGCTGAGATACGGGCTCCTAATCTCATGAA 1000
|||||
913 acagagaccaccagcaccaccctgagatacgggctcctaattctcatgaa 962
|||||
1001 ATACCCAGAGGTTGAAGAGAACTTCATGARGAAATGACAGGGTCATTG 1050
|||||
963 ataccagaggttgaagagaaacttcataaagaaattgacagggtcattg 1012
|||||
1051 STCCAACAGAAATCCCTGCCATCAAGGACAGGGCTGGACATGCCCTACCTG 1100
|||||
1013 gtccaaacagaatccctgccatcaaggacaggctggtcatgccctacctg 1062
|||||
1101 GATGCCGTGGTACATGAGATTCAGCGATTTCATCGACCTCATTCCTCCAA 1150
|||||
1063 gatgccgtggtacatgagattcagcgattcatcgacctcattccctccaa 1112
|||||
1151 CCTGCCACATGAAGCAACCCGGGACACAGTATTCAGAGACTACATCATCC 1200
|||||
1113 cctgccacatgaagcaaccgggacacagatttcagagactacatcatcc 1162
|||||
1201 CCAAGGGCACAGTGGTAATTCGACACTGGACTCCGTCTTATATGACAGC 1250
|||||
1163 ccaagggcacagtggtaattccgacactggactccgtcttatatgacagc 1212
|||||
1251 CAAGAATTCCTGAGCCGAGAGAGTTTAAAGCCAGAGCACTTCTGAATGA 1300
|||||
1213 caagaattcctgagccgagagagtttaagccagagcactttctgaatga 1262
|||||
46total.pair (744)

```

Fig. 6 (Continued)

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1301 AAACGGAAAGTTCAAGTACAGTGATCATTTCAGGCATTTCCGCAGGAA 1350
|||||
1263 aaacggaaagttcaagtacagtgatcatttcaaggcattttccgcaggaa 1312
|||||
1351 AGCGGGTGTGTGTCGGAGAGGGCCTGGCTCGCATGGAACGTTCCTGTTC 1400
|||||
1313 agcgggtgtgtgtcggaggggctggctcgcattggaagtgttcctgttc 1362
|||||
1401 ATGGCTGCCATCTTGACAGCACTTTAACTTGAAATCTCTTGTGACCCCAA 1450
|||||
1363 atggctgccatcttgacagcactttaacttgaaatctctgttgaccccaa 1412
|||||
1451 GGATATCGACCTCAGCCCCATCGGATTGGGTTTGCCAGATTCCCCCCC 1500
|||||
1413 ggatatacgacctcagccccatcggattgggtttgccagattccccccc 1462
|||||
1501 ATTACAAACTCTGTGTCAATCCCCGCTCACAAGTGTGAGGGAGATGTGCT 1550
|||||
1463 attacaaactctgtgtcattccccgctcacaagtgtagggagatgtgtc 1512
|||||
1551 CTAAAGGCCCTGGTTCCCTGATGCTGACCTGGAGGCCCTCTGTCCCCAGT 1600
|||||
1513 ctaaaggccctggttccttgatgtgtgacctggaggcctcctgtcccaqt 1562
|||||
1601 GTCCCCACAGGGAGCGCAGCCCGGGCTCCATAGGAAATCAAATGGGCCAG 1650
|||||
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|||||
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|||||
1613 tgaagctgcttcagccacatccttcagatagaattgaaagcaaagtc 1662
|||||
1701 CAAAAAGATTTTGTACAATCAATTAAAGTAAGTAAAGCCAAAAA- 1750
|||||
1663 caaaaaagattttgtacaatcaattaaagtaagtaagtc..... 1702

```

```

spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%

```

Fig. 6 (End)

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1MTALGITVALLVWLVTLLLLISINWKHIHSSWKLPFGPFPPLPIVGNIF 46 - SEQ ID No. 2
 1 RHEAMTALGITVALLVWLVTLLLLISINWKHIHSSWKLPFGPFPPLPIVGNIF 50 - AB000885

47 QLDLKNIPKSFMTLAERYGPVFTVYLGSRRIVVHLGYKAVKEVLLHYKNE 96
 51 QLDLKNIPKSFMTLAERYGPVFTVYLGSRRIVVHLGYKAVKEVLLHYKNE 100

97 FSGRGEIPTFQVHKDKGVIFNNGPTWRDTRRFSLTTLRDFGMGKQNEOR 146
 101 FSGRGEIPTFQVHKDKGVIFNNGPTWRDTRRFSLTTLRDFGMGKQNEOR 150

147 IQREAHFLEALRKTHGQPFDPFTFLIGCAPCNVISDILFRQHFDYNDKTC 196
 151 IQREAHFLEALRKTHGQPFDPFTFLIGCAPCNVISDILFRQHFDYNDKTC 200

197 LRLMSMFNENFYLLSTGWIQLYNNFSGYLRYLPGSHRKLKMKNISEIKDYA 246
 201 LRLMSMFNENFYLLSTGWIQLYNNFSGYLRYLPGSHRKLKMKNISEIKDYA 250

247 LERVKDHRDSLEPSCPRDETDTLLMEMEKEKYSAEPTITLDNIATVADM 296
 251 LERVKDHRDSLEPSCPRDETDTLLMEMEKEKYSAEPTITLDNIATVADM 300

297 FFAGTETTSTTLRYGLLILMKYFEVEEKLHEEIDRVIGPNRIPAIKDRLD 346
 301 FFAGTETTSTTLRYGLLILMKYFEVEEKLHEEIDRVIGPNRIPAIKDRLV 350

347 MPYLDVAVVHEIQRFIDLIIFSNLPHEATRDTFRDYIIPKGTVVVIPTLDSV 396
 351 MPYLDVAVVHEIQRFIDLIIFSNLPHEATRDTFRDYIIPKGTVVVIPTLDSV 400

397 LYDSQEFPEPEKFKPEHFLNENGKFKYSDFKAFSAGKRVCVGEGLARME 446
 401 LYDSQEFPEPEKFKPEHFLNENGKFKYSDFKAFSAGKRVCVGEGLARME 450

447 LFLFMAAILQHFNLSLVDPKIDILSPFAIGFAKIPPHYKLCVIER SQV* 496
 451 LFLFMAAILQHFNLSLVDPKIDILSPFAIGFAKIPPHYKLCVIER SQV* 500

497 GRCALKALVP*C*PGGLSPVSPQGAQPLHRKSNQFVKLLPAHILQIEF 546
 501 GRCALKALVP*C*PGGLSPVSPQGAQPLHRKSNQFVKLLPAHILQIEF 550

547 ESKV..... 550
 551 ESKVQKRFCIN*SK*8. 567

spice 23%
 spice 23%
 spice 23%

Fig. 7

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1 GCACACATTG AAAGATCCCC TGAAGGAGGC - SEQ ID No3

51 ATGACTGCCC TGGGCATCAC GGTGGCCCTG CTGGTGTGGT TGGTCACCCCT

101 GCTGCTCATC TCCATCTGGA AGCACATCCA CAGTAGGTGG AAACCTCCCC

151 CTGGCCCTTT CCCACTGCCC ATCGTTGGGA ACATTTTCCA GTTGGACCTT

201 AAGAATATTC CCAAAATCCTT CACCATGCTG GCAGAGCGTT ACGGGCCGGT

251 GTTCACTGTG TACCTGGGTT CGCGGCGCAT TGTGGTCTTG CACGGCTACA

301 AGGCCGTGAA GGAGGTCTTG CTCCACTACA AGAATGAGTT CTCTGGCAGA

351 GGGGAAATCC CCACGTTCCA AGTGACAAAG GACAAAGGGG TCATTTTCAA

401 TAATGGACCA ACCTGGCGGG ACACCTGGCG GTTCTCCCTC ACCACCCTCC

451 GTGACTTCGG GATGGGGAAA CAGGGCAATG AGCAGCGGAT CCAGAGGGAG

501 GCCCACTTCC TGCTGGAGGC ACTCAGGAAG ACCCATGGCC AGCCCTTTGA

total (334)

551 TCCACACTTC CTCATCGGCT GCGCACCTTG CAATGTCATC TCCGACATCC

601 TCTTCCGCCA GCACCTTGAC TACATGACA AGACCTGTCT GAGGTTAATG

651 AGCATGTTCA ATGAGAACTT CTACCTGCTC AGTACCGGCT GGATCCAGCT

701 TTATAATAAT TTCTCAGGCT ATCTACGCTA CCTGCCTGGA AGCCATAGGA

751 AACTAATGAA AATATATCT GAAATAAAG ATTATGCTTT AGAAAGAGTG

801 AAGGACCACC GGGATTCAC TGGAGCCAGC TGTCTCGAG ATTCACTGA

851 CACCCTGCTG ATGGAAATGG AGAAGGAAAA ATACAGTGCA GAACCTATAT

901 ACACCTTGA CAACATTGCC GTGACCGTGG CCGACATGTT CTTTGGGGG

951 ACAGAGACCA CCAGCACCAC CCGAGATAC GGGCTCCTAA TTCTCATGAA

1001 ATACCCAGAG GTGAAGAGA AACTTCATGA AGAAATTGAC AGGCTCATTG

1051 GTCCAAACAG AATCCCTGCC ATCAAGGACA GGCTGGACAT GCCCTACCTG

total (634)

Fig. 8

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1101 GATGCCGTGG TACATGAGAT TCAGCGATTG ATCGACCTCA TTCCCTCCAA
1151 CCTGCCACAT GAAGCAACCC GGGACACAGT ATTCAGAGAC TACATCATCC
1201 CCAAGGGCAC AGTGGTAATT CCGACACTGG ACTCCGTCTT ATATGACAGC
1251 CAAGAATTCC CTGAGCCGGA GAAGTTTAAG CCAGAGCACT TTCTGAATGA
1301 AAACGGAAAG TTCAAGTACA GTGATCATTT CAAGGCATTT TCCGCAGGAA
1351 AGCGGGTGTG TGTCCGAGAG GGCCTGGCTC GCATGSAACT GTTCCTGTTC
1401 ATGGCTGCCA TCTTGCAGCA CTTTAACTTG AAATCTCTTG TTGACCCCAA
1451 GGATATCGAC CTCAGCCCCA TCGCGATTGG GTTTGCCAAG ATCCCCCCCC
1501 ATTACAAACT CTGTGTCAAT CCCCCTCAC AAGTGTGAGG GAGATGTGCT
1551 CTAAAGGCCC TGGTTCCTTG ATGCTGACCT GGAGGCCTCC TGTCCCCAGT
1601 GTCCCCACAG GGAGCGCAGC CCGGGCTCCA TAGGAAATCA AATGGGCCAG
5total (93%)

1651 TGAAGCTGCT TCCAGCCCAC ATCCTTCAGA TAGAATTGA AAGCAAGTC
1701 CAAAAAAGAT TTTGTACAAT CAATTAAAGT AAGTAAAGCC AAAAAAAAAA
1751 AAAAAAAAAA AAAAAAAAAA

Fig. 8 (Continued)

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1QFFDPTFLIGCAPCNVISDILFRQHFDYNDKTC 33 - Part of
 ||||| SEQ ID No. 4
 151 IQREAHFLEALRKTHGQPPDPTFLIGCAPCNVISDILFRQHFDYNDKTC 200 - SEQ ID No. 5
 ||||| AB 000885
 34 LRIMSMENENFYLLSTGWIQLYNNFSGYLRLPGSHRKLKMNISEIKDYA 83
 |||||
 201 LRIMSMENENFYLLSTGWIQLYNNFSGYLRLPGSHRKLKMNISEIKDYA 250
 |||||
 84 LERVKDHRDSLEPSCPRDFTDTLLMEMEKEKYSABPIYTLDNIAVTVADM 133
 |||||
 251 LERVKDHRDSLEPSCPRDFTDTLLMEMEKEKYSABPIYTLDNIAVTVADM 300
 |||||
 134 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGENRIPAIKORLD 183
 |||||
 301 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGENRIPAIKORLV 350
 |||||
 184 MPYLDVVHEIQRFIDLIPSNLPHEATRDTVFRDYIIPKGTUVIPTLDSV 233
 |||||
 351 MPYLDVVHEIQRFIDLIPSNLPHEATRDTVFRDYIIPKGTUVIPTLDSV 400
 |||||

spice 9%
 spice 9%

Fig. 9

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1 ...GTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLD 47 - SEQ ID N6.6
|||||
301 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLV 350 - AB000885
48 MPYLDVVHEIQRFIDLIPSNLPHEATRDTVFRDYIIPKGTVVVPTLDSV 97
|||||
351 MPYLDVVHEIQRFIDLIPSNLPHEATRDTVFRDYIIPKGTVVVPTLDSV 400
98 LYDSQEFPEPEKFKPEHFLNENGKFKYSDHFKAFSAGKRVCVGEGLARME 147
|||||
401 LYDSQEFPEPEKFKPEHFLNENGKFKYSDHFKAFSAGKRVCVGEGLARME 450
148 LFLFMAAILQHFNLKSLVDPKDIDLSPITIGFAKIPPHYKLCVIPRSQV. 196
|||||
451 LFLFMAAILQHFNLKSLVDPKDIDLSPITIGFAKIPPHYKLCVIPRSQV* 500

Fig. 10

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